The Quantification of the Drug Gabapentin in Tissue Samples by HPLC-ESI-TOF-MS

Research Thesis

Presented in partial fulfillment of the requirements for graduation "with research distinction in Chemistry" in the undergraduate colleges of The Ohio State University

By

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Abstract

Gabapentin is being studied for use after high-level spinal cord injury (above thoracic level five) to prevent abnormal neuron sprouting and aberrant synaptogenesis which causes a life threatening condition Autonomic Dysreflexia (AD). Without treatment, AD causes high blood pressure which can induce stroke, seizures, and even death. It is theorized that gabapentin will reduce swelling around the site of injury which will enhance axon growth and plasticity culminating in improved recovery of neurological function after spinal cord injury. High performance liquid chromatography electrospray ionization time-of-flight mass spectroscopy (HPLC-ESI-TOF-MS) was used for the quantification of gabapentin in tissue serum samples. Solutions of samples were prepared and concentrations of drug were determined on the basis of calibration curves created using standards prepared. The samples were spiked with an internal standard directly after tissue harvest and prior to any further manipulation to correct for variations in sample preparation and analysis. This separation and detection method is suitable for quantitative sample analysis at low concentrations. With the developed method, it has been shown that HPLC-ESI-TOF-MS is capable of determining the concentrations of gabapentin in serums. This method of detection will assist neuroscientists in the determination of efficacious dosages as well as administration methods.

In Memory of William Edward Beinlich

Acknowledgments

I wish to give a special thanks to J. Clay Harris, who coordinated this project collaboration, for his continued support, patience, and advice throughout my undergraduate career. I would also like to thank Noel M. Paul for his unwavering guidance in meeting and tackling new challenges. To my family and friends, thank you for pushing me to be my best and supporting me along the journey.

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1. Introduction

1.1 Gabapentin

Gabapentin, 1-(aminomethyl-1-cyclohexyl)acetic acid (GBP) (Figure 1), is an antiepileptic drug designed from ϒ- aminobutyric acid (GABA). GBP is able to cross the blood-brain barrier and is employed for the treatment of partial and generalized tonic-clonic seizures in adults and children.[1,2] It has also been shown to ease chronic neuropathic pain.[3] More recently, it has been shown as an anti-epileptic/anti-neuropathic pain drug, gabapentin (Neurontin^{TM}) binds with α2δ-1 thereby blocking thrombospondins (TSP)/neuronal α2δ-1 interactions as well as inhibiting TSP-induced new synapse formation.[4,5] By inhibiting TSP binding to neuronal, it is hypothesized that gabapentin will reduce the severity and frequency of Autonomic Dysreflexia (AD) by inhibiting maladaptive synaptogenesis after spinal cord injury.

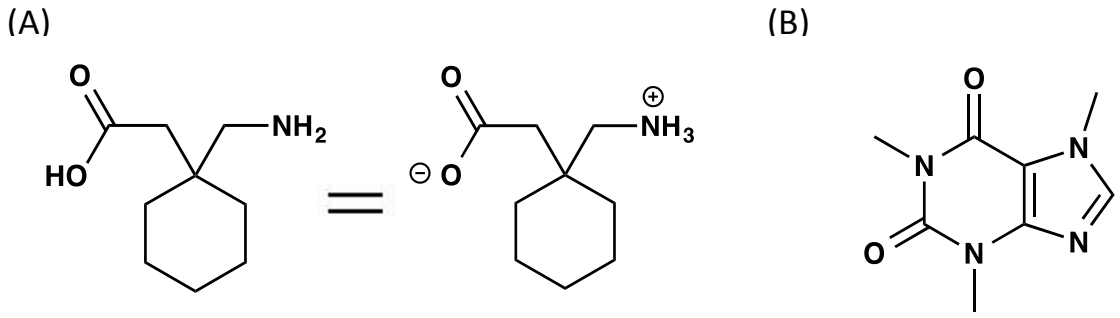


Figure 1: chemical structures of (A) gabapentin in balanced neutral and zwitterionic form and (B) internal standard (caffeine)

GBP is currently under investigation by The Ohio State University Department of Neurology for use after high-level spinal cord injury (above thoracic level five (T5)) to prevent abnormal neuron sprouting and aberrant synaptogenesis which causes AD. AD is a life threatening condition of episodic vascular hypertension (often with bradycardia, or slowed heart rate) that develops in most (approximately 90%) of the 300,000 new cases a year of spinal cord injury (SCI) above thoracic spinal

level T5. [6,7] AD is an abrupt, uncontrolled sympathetic response caused by stimulus below the level of injury and can lead to severe hypertension, cerebral hemorrhage, and death.[8] After high level SCI, loss of supraspinal control together with aberrant collateral sprouting and formation of new intraspinal synapses causes spinal autonomic reflexes to become exaggerated. [9,10] This post-injury maladaptive neural plasticity, involving sensory axons and propriospinal interneurons that connect multiple segments of the thoracic and upper lumbar spinal cord, progresses slowly over the course of several weeks or months post-injury. This results in an increased blood pressure response during bladder distention or other common stimulus below the level of injury in SCI patients. [11]

Prevention (e.g., regular bladder/bowel voiding) and anti-hypertensive medications are currently the best way to "manage" AD; however, there is no cure.[12] In this study, concentrations of GBP will be quantified in spinal cord samples of mice in order to determine a dosage and delivery method to use on a full scale experiment where genetic and pharmacological tools will be used to test the hypothesis that post-injury inhibition of reactive synaptogenesis will block the onset or reduce the severity of AD.

GBP's bioavailability may vary greatly between subjects because of its particular active absorption by the gut and excretion by the kidney due to dose- dependent oral absorption kinetics and dose- independent disposition kinetics. [13,14] It is also not known in what concentrations GBP will present itself in the spinal cord for this treatment purpose given different delivery methods and how it will be digested in the body for dosage purposes. It is for these reasons that the quantification of GBP in spinal cord samples will be useful to neurology researchers in evaluating GBP dosage and delivery methods before moving to a full scale experiment of use in post- spinal injury cases.

Although gabapentin is currently widely used for seizures and neuropathic pain management, its bioavailability is still not well understood. Many analytical techniques have been implemented and reported for the quantitative determination of gabapentin in tissue, plasma, or serum including: high-

performance liquid chromatography (HPLC) with UV detection[15], HPLC coupled with electrospray tandem mass spectroscopy (ESI-MS)[16], liquid chromatography tandem MS (LC-MS/MS)[17], gas-liquid chromatography (GLC) with flame ionization (FI)[18], and capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection[19]. However, using conventional HPLC, GC, or CE methods require long work up processes to extract GBP from biological samples.

HPLC with UV detection method required a reversed-phase solid-phase extraction which involves placing plasma samples in a glass culture tube with an internal standard and buffer followed by activation of a solid-phase extraction cartridge and finally passing the mixture through a conditioned cartridge by gravity followed by buffer and acid washes and then eluting the cartridge with methanol. This work up process is then followed by a derivatization which includes base which is added to the eluent and dried under nitrogen to which a derivatizing agent, phenylisothiocyanate in this case, was added to react with the residue for 20 minutes which was then dried under nitrogen again. The sample went on to be reconstituted with mobile phase and then analyzed with a run time of 8 minutes and a detection limit of 30 ppb. [15] GLC-FI determination of gabapentin in serum also required a lengthy workup procedure before the samples were able to be run. The method required an extraction of acidified sample by solid- phase column, derivatization (agent added then solution put into a water bath (70 °C) for 30 minutes, then cooled 10 minutes), and then analysis on an HP-1 column (5 minutes) with FI detector. This resulted in a limit of detection of 200 ppb with a limit of quantification of 500 ppb, linear range from 500 ppb to 30 ppm. [18] The CE-LIF analysis method also required a derivatization procedure using 6-carboxyfluorescein succinimidyl ester vortexed with the sample for 30 minutes resulting in a limit of detection of 10.3 ppb (60nM). [19]

This is in direct contrast to the sample preparation required to run an HPLC-ESI-MS. This analysis method requires the sample to be vortexed (20 seconds) with the internal standard before acetonitrile is added and vortexed again (20 seconds) then centrifuged (10 minutes) when the upper layer is then

removed and diluted with mobile phase before being run with a run time of 4 minutes with a lower limit of quantification of 50 ppb in the linear range (50 ppb- 10 ppm).[16] This extraction process closely mimics the HPLC- MS/MS method used which also included a simple protein precipitation workup before samples were run with a limit of detection of 10 ppb and a lower limit of quantification of 40 ppb with a run time of only 2 minutes.[17]

The required derivatization of samples to extract gabapentin from biological samples is due to its zwitterionic characteristic (Figure 1A). The additional work-up steps are to produce a chromophore so it can be analyzed using the analytical techniques mentioned above. The extraction processes (multi-step liquid-liquid or solid-phase extractions) all increase sample preparation time as well as the cost of the method. They also do not produce the most precise results as their limits of detection are larger than LC-MS methods mentioned above. For this reason, a high performance liquid chromatography electrospray ionization time-of-flight mass spectroscopy (HPLC-ESI-TOF-MS) method was employed to quantify GBP quickly, accurately, and at a lower long term cost than previous methods as the sample preparation does not require such extensive extraction processes.

1.2 Analysis

The samples of tissue serum are introduced into high performance liquid chromatography (HPLC) analysis by an autosampler. The HPLC serves to separate the biological sample into various groups of molecules based on the molecules characteristics such as polarity and size. This occurs due to different interactions between the stationary phase and mobile phase of the column.

The HPLC is coupled to Agilent Jet Stream Electrospray Ionization (AJS ESI) technology, which will operate in positive mode for this purpose, and is a very sensitive ion source for this sample type. It incorporates high velocity heated gas to generate large amounts of analyte ions while including an

internal mass reference spray (IRM). The sample ions are related to the IRM ions from within each individual scan. Using the relative mass differences provides very accurate (<2ppm accuracy) and precise (down to 100 Daltons mass range) mass measurements.[20]

The ESI creates positively charged ions (Figure 2) for analysis by nebulizing the IRM and sample solution flow from the HPLC into the ESI spray chamber using nitrogen as the nebulizing gas. The droplets in the spray chamber are dried using heated nitrogen which evaporated the droplets until they reach the Rayleigh limit in which the positive charge builds up on the droplet such that the droplet goes through a Columb explosion. These smaller droplets are dried again and again in the chamber until just a positively charged analyte ion is left.

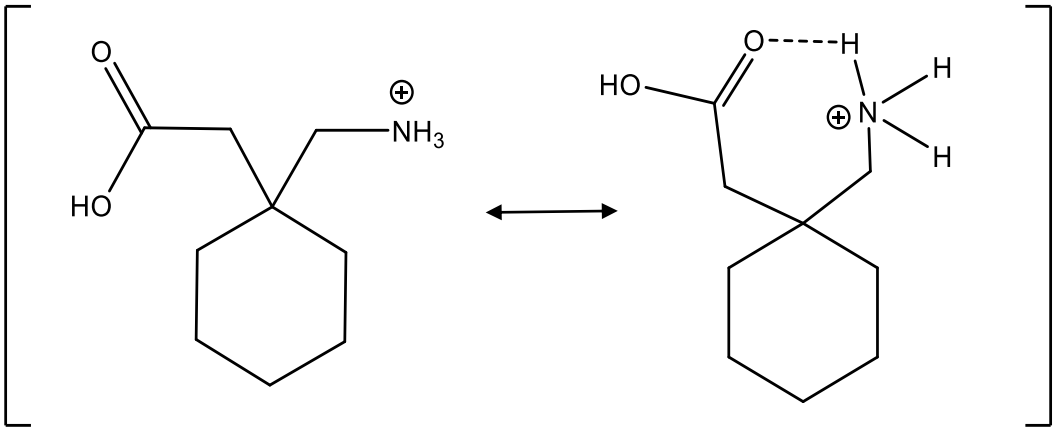


Figure 2: Proposed chemical structures of protonated gabapentin

A dielectric platinum-plated capillary separates the spray chamber from the ion optic region. A voltage is applied to the front end of the capillary (Vcap) in order to maximize transmission of the positively charged ions formed in the atmospheric spray chamber as they travel through the capillary to the lower pressure ion optic region where they are focused and introduced into the mass analyzer.[21]

The pressure drop exiting the capillary creates a supersonic beam that produces a Mach disk between the capillary and the first skimmer. The skimmer serves to sample the ions, putting the

skimmer after the Mach disk increases sensitivity before the ion proceeds into the octopole. The octopole reduces the ion energy distribution before mass analysis and consists of eight rods with RF voltage in addition to a DC offset voltage (adjacent rods have opposite polarity voltages).[22] Finally, the ion beam goes through two lenses before entering the mass analysis portion of the instrument. The first lens serves as an electrostatic lens to focus the ions and the second lens has an RF voltage applied to it which pumps away most of the excess neutrals.[23]

Once in the flight tube, the ions are pushed into flight by an ion pulser. The ion pulser is made of a stack of plates, each (except the last, back plate) with a hole in the center. The ions enter the stack of plates between the back plate and the first plate with a hole in the center. A high voltage is applied to the back plate which accelerated the ions through the holes in the stack of plates to create a beam of ions.[24] The beam of ions fly up into the flight tube.

At the end of the flight tube there is a two stage ion mirror. This ion mirror utilizes plates of increasing electrical potential to slow ions as they penetrate deeper into the plates on the top of the light tube (Figure 3). This increases the accuracy of mass measurements as spatial and kinetic energy distributions (Figure 4) are minimized.

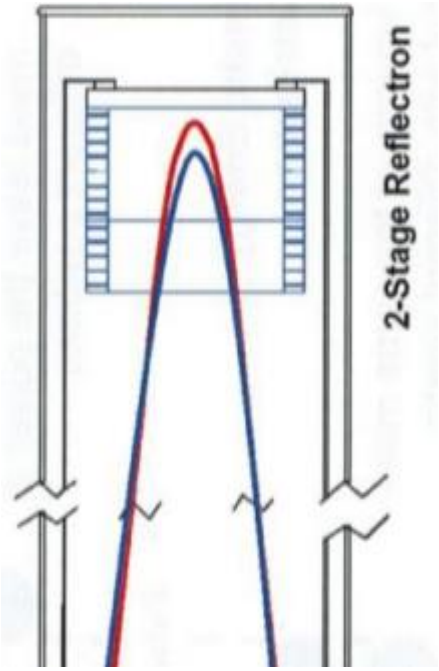


Figure 3: Ion mirror equalizes arrival time of two ions of the same mass at the detector[25]

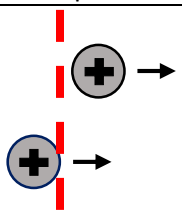
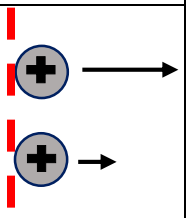
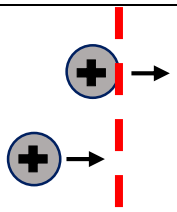
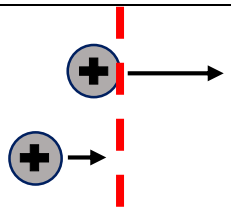
	Spatial Distribution- two ions of the same mass leave the pulser at different positions	**Kinetic Energy Distribution**- two ions of the same mass but different kinetic energies.
Pulser		
Detector		

Figure 4: Variation in flight time illustrated

The ions then reach the surface of the ion detector due to a charged surface used to attract the ions. The surface of the ion detector is comprised of microscopic tubes that pass from the surface to the

rear of the microchannel plate (MCP). When the ion strikes the surface, an electrical signal amplification process takes place. When the MCP is struck, an electron becomes free and hits the walls of a microscopic tube which causes more electrons to become free and this continues as an increasing number of electrons travel to the rear of the plate through the microscopic tube. These electrons are then focused on a scintillator which produces a flash of light when struck by electrons. This light is then focused through two small lenses onto a photomultiplier tube (PMT). This electrical signal is then read by the data system. An optical signal is created from the MCP signal because the MCP output is approximately -6000 V while the PMT output is at ground potential.[26]

Fundamentally, a time of flight mass spectrometer (TOF-MS) measures time, not mass. It employs fundamental laws to calculate mass from the time of flight.

It is widely accepted that the speed of an electron is proportional to the voltage applied in acceleration (Equation 1).

$$eV = \frac{1}{2}mv^2$$

e= charge of an electron
V= voltage applied in acceleration
m= mass of an electron
v= speed

Equation 1: Speed of an electron.[27]

The speed is proportional to the length of the path traveled and the time it took to travel that length (Equation 2).

$$v = \frac{L}{t}$$

v= speed
L= length of flight path
t= time

Equation 2: Speed is proportional to length and time of path traveled.[27]

One can combine the above equations one and two to give an equation that gives us an equation of time based on the mass and charge of an ion (Equation 3).

$$t = L\sqrt{\frac{m}{2eV}}$$

Equation 3: time calculated from length of flight path, mass and charge of an electron, and voltage applied in acceleration.[27]

One is able to measure the time of flight in the TOF-MS. The length of the flight tube is consistent and known and the voltage applied in acceleration is known. One can then measure the mass-to-charge ratio of the analyte based on equation four.

$$\frac{m}{z} = \left(\frac{2V}{L^2}\right)t^2$$

Equation 4: Mass to charge ratio calculated from time, length of flight path, and voltage applied.[27]

The flight tube is also under high vacuum (9.6×10^{-8} Torr) so no other particles interfere with the flight pattern. The TOF pulse plate pulses ions into flight about every 100 microseconds while the detector takes a measurement every one nanosecond, equivalent to one billion spectra per second. There are 100,000 data points in each transient. This all equates to spectra with excellent ion statistics.[28]

2. Methodology

2.1 Materials

Gabapentin (G154) was obtained from Sigma- Aldrich (St. Louis, MO). Sterile latex-free syringes, 1 mL norm-ject tuberkulin, were purchased from Henke Sass Wolf (Tuttlingen, Germany) with sterile precision glide needles 22G1½ from Becton Dickinson & Co. (Franklin Lakes, NJ, USA). Microvials used for sample analysis were purchased from MicroSolv Technology (Eatontown, NJ, USA). Captiva regenerated cellulose syringe filters (pore size 0.2 µm, Product: 5190-5106, Lot: 1308004VS) were

purchased from Agilent Technologies (Wilmington, DE, USA). HPLC grade acetonitrile with 0.1 % formic acid (Product: HB9823-4, Lot: 143003), HPLC grade water with 0.1 % formic acid (Product: HB523-4, Lot: 138713), and ACS grade (99.7%) caffeine (Product: AA39214-14, Lot: G31Z005) were purchased from Fisher Scientific (Ann Arbor, MI, USA). HPLC ready deionized 18 MΩ-cm water was obtained, in-house, from a Barnstead NANOpure infinity base unit analytical version model D8961, Barnstead/ Thermolyne Corp., (Dubuque, IA, USA).

2.2 Instrumentation and conditions

An Agilent 1290 Infinity LC Injector HTC (CTC PAL), (Agilent Technologies, Wilmington, DE, USA), autosampler introduced the samples into the HPLC-ESI-TOF-MS system. Wash solvent A was acetone in water with 0.1 % formic acid and wash solvent B was 1:1:1 acetonitrile-dichloromethane-methanol. The wash solvents were used to wash both the inside and the outside of the injection needle between each injection to prevent sample cross-contamination. A sample volume of 5 µL was used during the injection. The aliquot was used to first flush the sample loop in the six-port injection valve before the 2 µL sample loop was filled and the sample was injected into the instrumentation.

The HPLC 1290 Infinity LC system consists of a Hewlett Packard series (Agilent Technologies, Wilmington, DE, USA) equipped with a Jet Weaver V35 mixer, a G1316C thermostatted column compartment (TCC), and G4212A diode-array detector (DAD). A Zorbax Eclipse Plus C18 Rapid Resolution HD (RRHD) column, 2.1 mm x 50 mm, 1.8 µm (Product: 959757-902, Lot: B11048) fitted with a filter installed ahead of the column was used in the separation method. The elution was an optimized gradient between solutions A (5% acetonitrile in water) and solution B (95% acetonitrile in water). A linear gradient from 95:5 to 5:95 A-B for 9.5 minutes followed by isocratic 5:95 A-B conditions for 2.0 minutes and a gradient back to 95:5 A-B conditions for the last 0.5 min all at 0.100 mL/min flow rate.

The column pressure was limited to 600 bar and temperature was maintained at 20 °C. The column eluent was analyzed by TOF-MS, using positive ESI.

The nitrogen used as the drying gas in the ESI-TOF was obtained in house from a Parker Nitroflowlab, Parker Filtrations & Separation B.V. (Etten-Leur, Netherlands). It was at minimum 98 % pure and introduced into three big universal traps in parallel before entering the ESI. The nitrogen drying gas was pumped into the nebulizer at 25 psig at 9 L/min. It was also used as the sheath gas flowing at 8 L/min.

An ESI-TOF-MS 6230A (Figure 5) (Agilent Technologies, Wilmington, DE, USA), was run in positive mode and a fast switch capillary 0.6 mm (Product: G1960-80060) from Agilent was employed. The ion source was Agilent jet stream electrospray ionization (AJS ESI) with gas temperature at 325 °C. The VCap was set to 2750 V and the nozzle voltage was 500 V. The MS TOF fragmentor was 175 V, the skimmer was 65 V, and the octopol 1 RF Vpp was 750 V. The TOF spectra was collected in the range of 100 m/z to 1000 m/z at an acquisition rate/time of 3 spectra/second and time 333.3 ms/spectrum. The transients/spectrum was 3285. Both a TIC and BPC chromatograms were collected. While the samples ran, a reference mass solution was also pumped through the TOF-MS with references masses of 118.086255 m/z and 922.009798 m/z at a detection window of 100 ppm with a minimum height of 400 counts.

All instrumentation was ran with Agilent MassHunter workstation software LC/MS data acquisition for 6200 series TOF, version B.05.01, Build 5.01.5125.1.

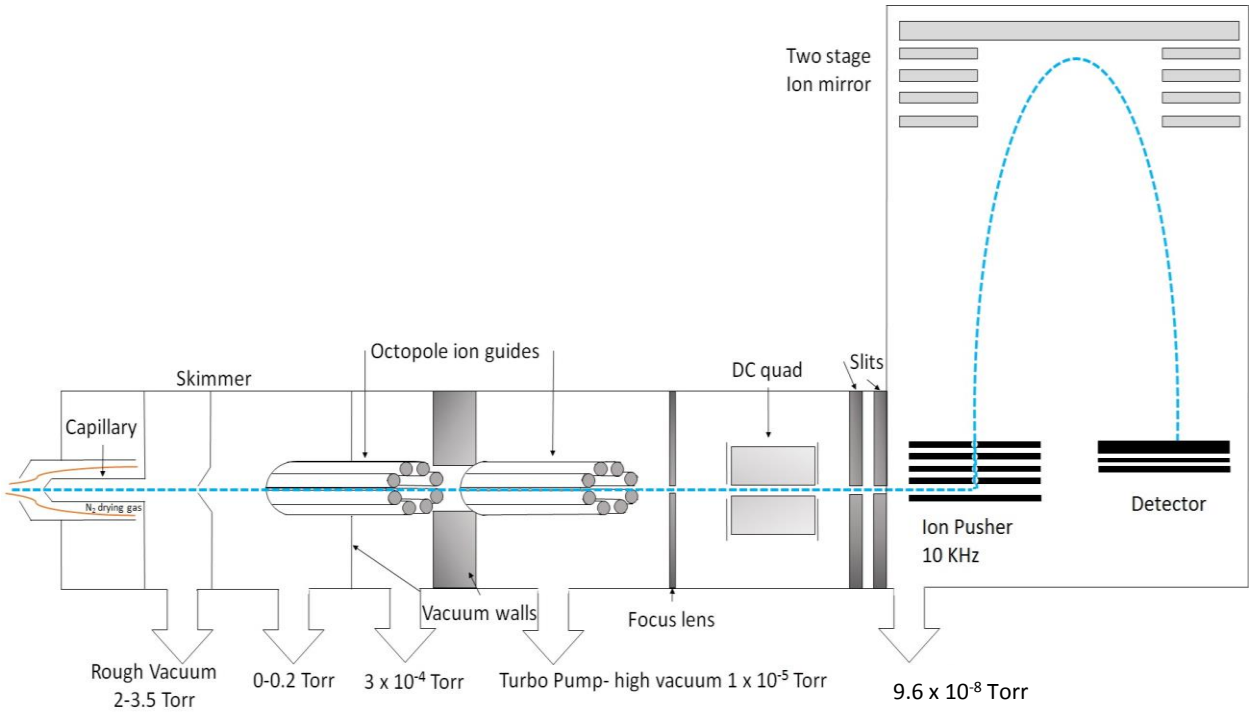


Figure 5: TOF-MS diagram

3. Experimental Procedure

3.1 Preparation of internal standard solution

A batch of 10 ppm caffeine solution was prepared in lab and provided to our collaborator, Dr. Yan Wang, Department of Neuroscience, to use as an internal standard for sample preparation.

3.2 Biological Preparation of Samples

All surgical and post-operative care procedures were performed in accordance with The Ohio State University Institutional Animal Care and Use Committee. Age- and weight-matched female C57BL/6 mice were obtained from Jackson Laboratories.

Mice were a part of one of four dosage and delivery groups or a control group (treatment code $G_{\circ}$). One delivery system was a mini pump which delivers the gabapentin in subcutaneous injections at a rate of 0.12 µg/day. Two subgroups were within the mini pump group which included administering the drug in a phosphate buffered saline (PBS) for either one week (treatment code 1) or three weeks (treatment code 2) post spinal injury. Another delivery system was investigated, hand injections subcutaneously with 10 mg/mL gabapentin in PBS solution with the dosage of 200 mg/kg/day (mg of drug/ kg of body weight) for 8 days. Two subgroups were within this delivery system as well. Mice either received the injections every 2 hours (treatment code 3) or every 32 hours (treatment code 4).

A sample of tissue (10-100 mg) was obtained and homogenized in 200 µL of internal standard solution of caffeine in water (10 ppm caffeine). A simple protein precipitation with acetonitrile (1600 µL) was implemented. The sample was then vortexed for 30 seconds then centrifuged at 13,000 xG for 10 minutes at 4 °C. The clear supernatant was then transferred to a clean 1.5 mL conical bottom tube and dried completely under a gentle stream of nitrogen gas. The dried samples were then stored at -80 °C until analysis.

3.3 Preparation of Samples for Analysis

The dried samples were brought to room temperature and reconstituted with 200 µL of nanopure water (18 MΩ-cm or greater). The samples were filtered through Agilent Captiva regenerated cellulose syringe filters (pore size 0.2 µm) into 300 µL autosampler microvials for analysis.

3.4 Creation of calibration curves

Standard solutions of gabapentin were created in water in the range of 0 ppb to 95 ppm gabapentin each spiked with the internal standard, 10 ppm caffeine. The solutions were analyzed

according to the above instrumentation and conditions. The responses from the known concentrations were used to create a calibration curve (Graph 1 and 2) (MassHunter Quantitative CurveFit Assistant) and used by the data analysis program to quantify the unknown concentrations of gabapentin in the biological samples.

3.5 Quantification method of drug in tissue sample

A basic quantification workflow was created in MassHunter Workstation software, Quantitative analysis version B.06.00 SP01, Build 6.0.388.1. A batch was created from analytical runs of calibration solutions and tissue samples. A method was created from the standards in the batch and information on the calibration levels and internal standard levels. The quantification method was verified within the program before being applied to the batch. The calibration curve fit options were explored to choose the best fit line. The integrations of the peaks were manually confirmed to verify the method created. Any messages and outliers that the program flagged were also addressed.

4. Results

4.1 MassHunter quantification method

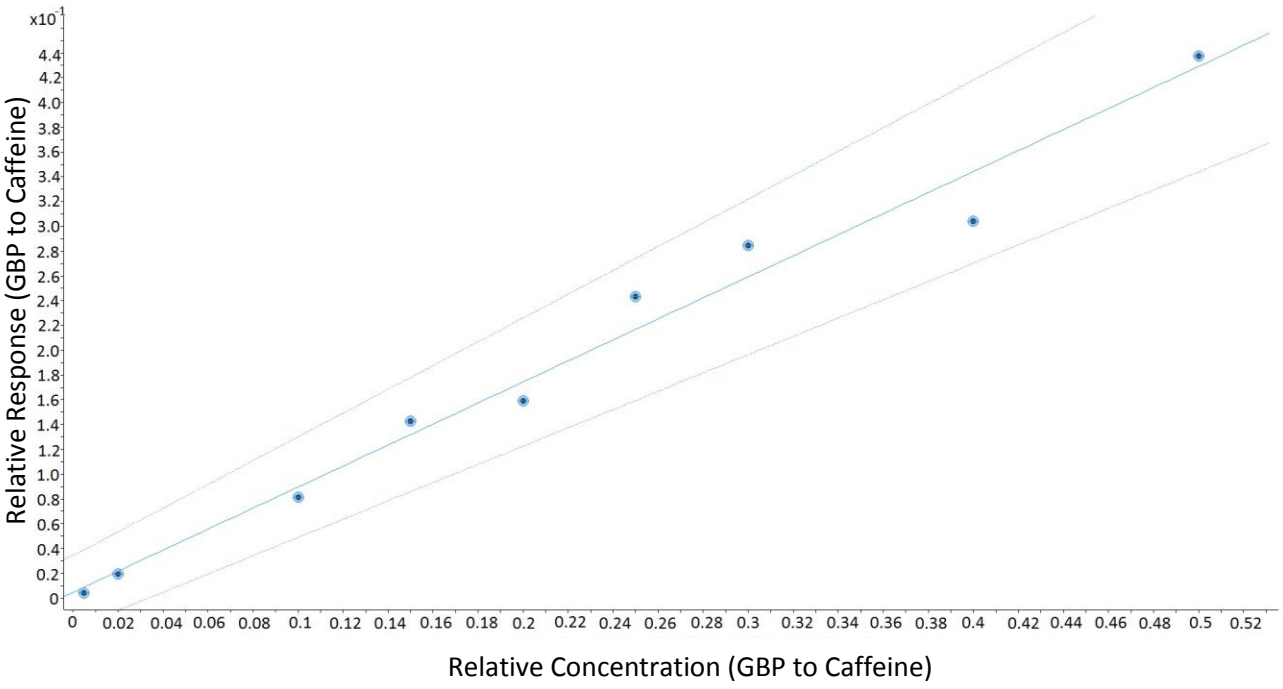
The method was developed with the help of the MassHunter Program. A new method used acquired data from a concentrated calibration solution (95 ppm) to create the method. The software identified quantifiers from the TIC scan as suggested ions to quantify. Both gabapentin and caffeine were identified from the TIC scan and were marked as quantifiers. The caffeine mass peak was identified as 195.0892 m/z (±0.0049 m/z: standard deviation of the mass measurement, calculated in appendix) and the gabapentin mass peak was identified at 172.1348 m/z (±0.0094 m/z: standard deviation of the

mass measurement, calculated in appendix). The caffeine was marked as the internal standard (ISTD in the program) and concentration was entered as 10,000 ppb as the program quantifies in unit terms of ppb (µg/L).

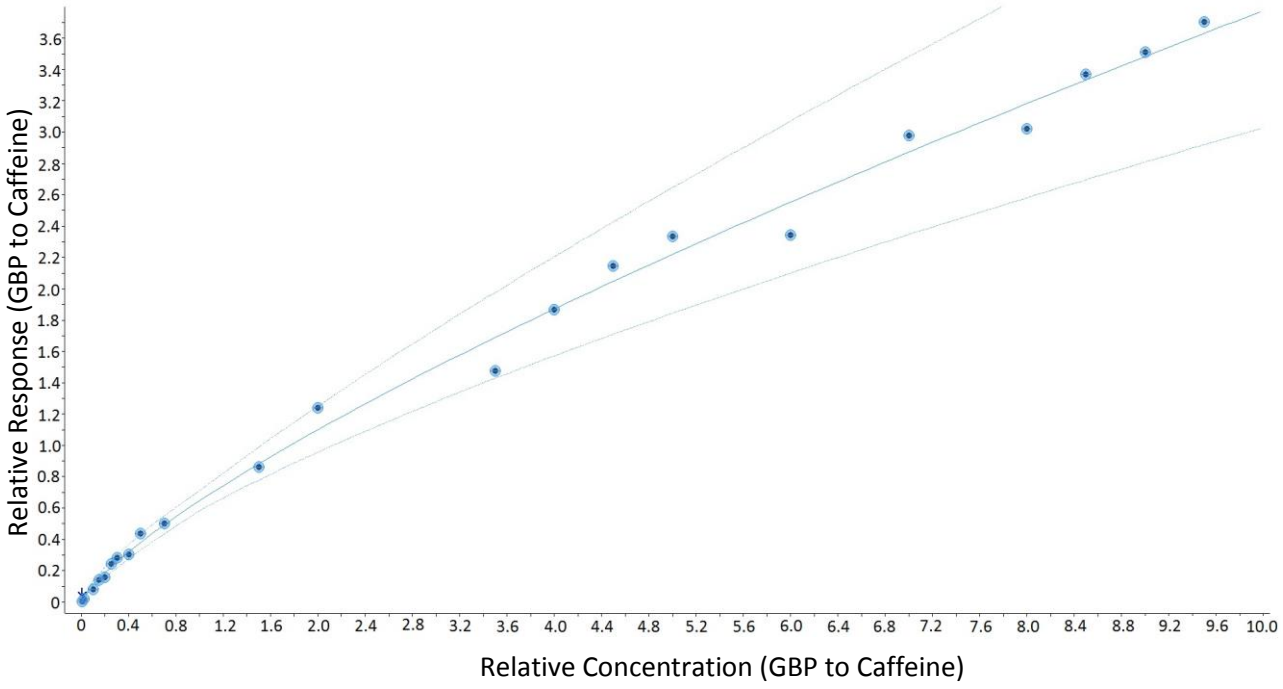
New calibration levels were added to include the calibration range from 0 ppb – 95 ppm, concentrations of GBP, with 10 ppm ISTD in each sample. The method was validated within the program and applied to analyze the batch of spinal samples and calibration solutions.

4.2 Calibration curve

A range of calibration solutions (0 ppb – 95 ppm) were used to create the calibration curve which was later used to quantify the spinal samples based on their response (counts). The calibration curve was found to be linear in the range of 0 ppb to 5 ppm ($y = 0.849085x + 0.004602$ (R^2=0.97902938)) (Graph 1) but behave in a power fashion in the extended range 0 ppb to 95 ppm ($y = 0.646127x^{0.766702}$ (R^2 = 0.99490159)) (Graph 2).



Graph 1: Linear Calibration curve in concentration range 0 ppb to 5000 ppb with confidence bands.



Graph 2: Power Calibration curve in concentration range 0 ppb to 95 ppm with confidence bands.

4.3 Verification of calibration curve

Calibration solutions were run as samples to verify the calibration curve and the MassHunter quantification method. A few calibration solutions across the concentrations were chosen for analysis. The verifications were run on different days along with the animal sample runs and the gabapentin quantifications (Table 1) were calculated with the animal samples using the same quantification method as the animal samples.

Name	Gabapentin Response (counts)	Calculated Concentration (ppb)	Accuracy	Within confidence limits?
Calibration test 1000 ppb gabapentin	4415361.907	1550	155%	Yes
Calibration test 1500 ppb gabapentin	4343230.052	1390	92.8%	Yes
Calibration test 2500 ppb gabapentin	7528907.739	2800	112%	Yes
Calibration test 7000 ppb gabapentin	9560688.645	7510	107%	Yes
Calibration test 35000 ppb gabapentin	28341692.07	34960	99.9%	Yes

Table 1: Calibration solutions run as samples for calibration curve verification

4.4 Drug quantification in spinal cord

Using the quantification method paired with the calibration curve, the spinal cord samples were examined for caffeine and gabapentin concentrations. The program identified and integrated the mass peaks and compared the counts of gabapentin peaks to the counts of the calibrated ISTD and the calibration curve. From this, the program was able to quantify the amount of gabapentin in each sample (Table 2).

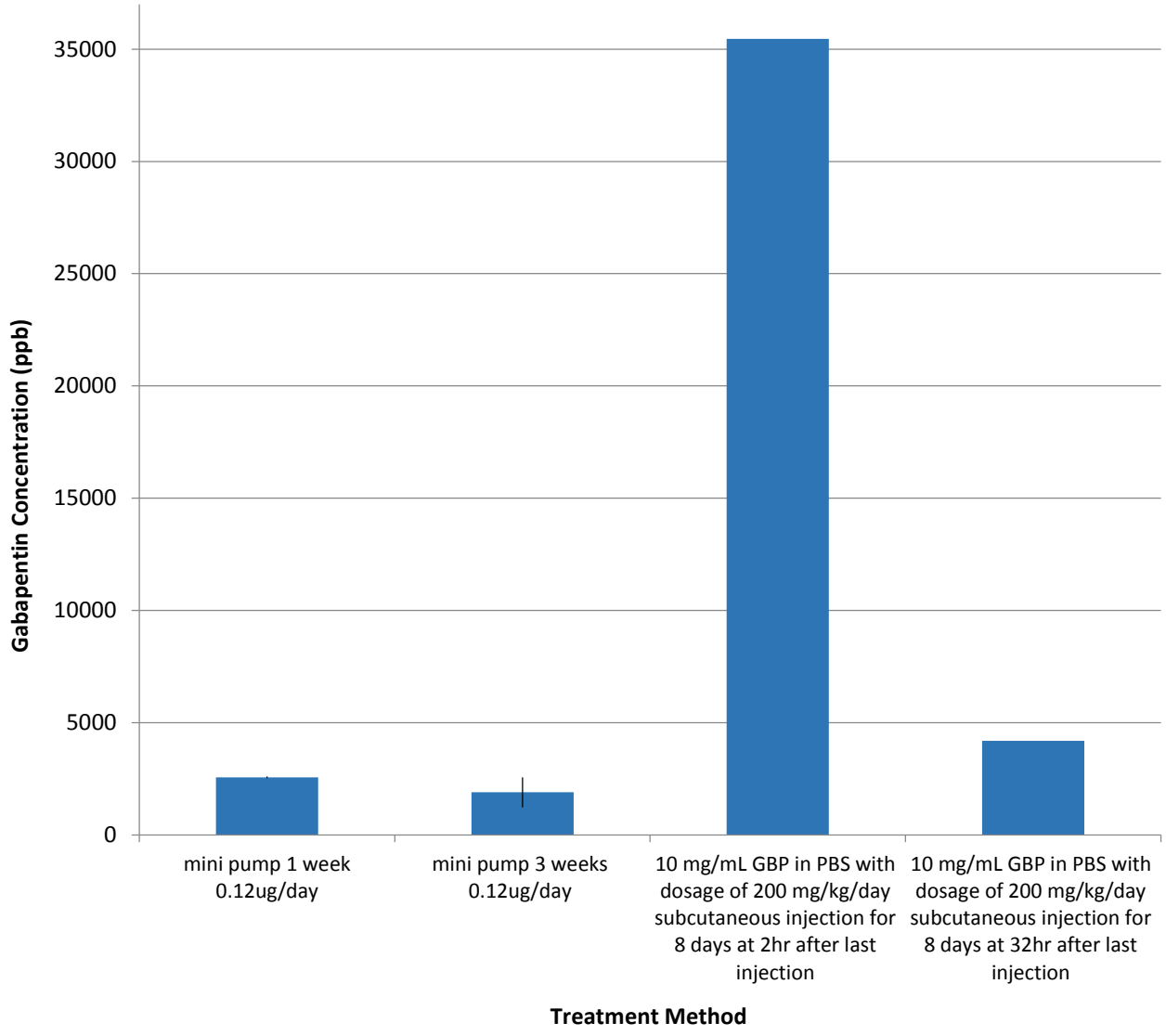
Name	Gabapentin Response (counts)	Calculated Concentration (ppb)
Gaba Ctrl SC	0	0 ±1030
Gaba AC Ctrl	0	0 ± 1030
Animal 1	534740.3723	850 ± 1030
Animal 2	644635.8086	2680 ±1030
Animal 3	810804.6474	2160 ±1030
Animal 4	1907171.441	33740 ±1040
Animal 5	872422.8221	2640 ±1030
Animal 6	633313.498	2530 ±1030
Animal 7	474312.2695	2540 ±1030
Animal 8	424773.1187	2470 ±1030
Animal 9	3659180.375	37190 ±1040
Animal 11	806740.9229	5930 ±1030

Table 2: Gabapentin concentrations in each animal sample.
Animal 10 sample was damaged in preparation and not included in the drug quantification process.

These values were obtained in a blinded experiment method in which the analytical chemist did not know which animals got what dosage of drug, which treatment method, or if they were treated at all. This form of experimentation is meant to withhold information not necessary to the step of the process in order to remove the possibility (intentional or unintentional) of the formation of a bias. The analytical chemist was informed of the test groups after the quantification was finalized for each animal sample, only then were the delivery and dosage methods investigated (Table 3) (Graph 3).

Name	Calculated Concentration (ppb)	Treatment Code	Treatment Method
Gaba Ctrl SC	0	G_o	None
Gaba AC Ctrl	0	G_o	None
Animal 1	850	2	mini pump 3 weeks 0.12ug/day
Animal 2	2680	2	mini pump 3 weeks 0.12ug/day
Animal 3	2160	2	mini pump 3 weeks 0.12ug/day
Animal 4	33740	3	200mg/kg/day subcutaneous injection for 8 days at 2hr after last injection
Animal 5	2640	1	mini pump 1 weeks 0.12ug/day
Animal 6	2530	1	mini pump 1 weeks 0.12ug/day
Animal 7	2540	1	mini pump 1 weeks 0.12ug/day
Animal 8	2470	4	200mg/kg/day subcutaneous injection for 8 days at 32hr after last injection
Animal 9	37190	3	200mg/kg/day subcutaneous injection for 8 days at 2hr after last injection
Animal 11	5930	4	200mg/kg/day subcutaneous injection for 8 days at 32hr after last injection

Table 3: Gabapentin concentration paired with treatment method for each animal



Graph 3: Average gabapentin concentrations, with error bars, in animal samples per treatment method

5. Discussion

The internal standard caffeine concentration was 10 ppm in each animal sample and calibration solution. After testing a range of caffeine concentrations (100 ppm, 50 ppm, 10 ppm, 1 ppm) 10 ppm was concluded to be the best option for analysis purposes. A concentration of 10 ppm caffeine was suitable due to a clear response peak without over saturating the detector or creating any ion

suppression of other analytes (Calibration investigation results in Appendix). The concentration 1 ppm had a narrow Gaussian shaped peak but only was detected at 250,000 counts at the peak. While this can be considered a good response, the 10 ppm concentration yielded approximately 3,700,000 counts at the signal peak which is more ideal. Concentrations of 50 ppm and 100 ppm also yielded response counts over 4 million, and while these did not over saturate the detector, too large of a count response can cause ion suppression of other analytes. The internal standard concentration of 10 ppm was also the most symmetric compared to the 50 ppm and 100 ppm concentrations. The 50 ppm and 100 ppm had tailing peaks and while some degree of tailing is unavoidable, performance of the data system starts to be compromised when the tailing becomes too large. It is for these reasons that the concentration of 10 ppm caffeine was used as the internal standard in this investigation rather than 1 ppm, 50 ppm, or 100 ppm. These values were evaluated in Agilent MassHunter workstation Qualitative analysis software, version B.06.00, Build 6.0.633.10, service pack 1 with bio confirm software.

The calibration curve is linear at lower concentrations of GBP but takes on a power series trend as the concentrations of GBP extend beyond 5 ppm to 95 ppm. Different proposals as to why the higher concentrations deviate from linearity have been proposed. The first is that there is a limited amount of excess charge available on ESI droplets to the point where analyte concentration can exceed the charges available.[29] A second conclusion to this trend is that there is limited space on a droplet surface and once the concentration approaches a certain limit, the surface becomes so saturated with analyte that ion ejection becomes inhibited because ions are trapped in the droplet interior.[30] A third possible cause is suppression and competition at high concentrations. For example, a surface-active analyte would be expected to out-compete polar analytes for the limited excess charge and/or space on the droplet surface as previously discussed.[31] Regardless of the reason, which is still not well established, the power series deviation from linearity is typical and expected for higher concentrations of analyte analysis.

As seen in the results (Table 1-2) there are a fractional number of counts of analyte ions as well as a large number of significant figures. Both the fractional counts and the large number of significant figures can be attributed to the detection method used in an HPLC-ESI-TOF-MS. The detector plate is a microchannel plate (MCP) comprised of many microscopic tubes or channels. The MCP is negatively charged to attract the positively charged analyte ions. When an ion hits the MCP, one or more electrons are released inside a channel which acts as an electron multiplier. Electrons cascade down the channel and exit at about ten electrons per ion that hit the surface of the MCP. When the electrons exit the channel, they are accelerated into a scintillator which emits photons when struck by electrons. The photons that are released from the scintillator are focused through optical lenses onto a photomultiplier tube (PMT). The PMT then produced an electrical signal proportional to the number of photons. The detection signal is converted from an electrical signal to an optical signal and then back to an electrical signal as to electrically isolate the flight tube and the detector which are roughly -6500 V, from the PMT, which outputs a signal at ground potential. [27]

During method development, it was noted that the column in the HPLC started to get contaminated by the high amounts of analytes that were run through the column. This issue was addressed by including an additional two minutes of run time to the end of each sample run that served as a wash between sample runs. This built in wash was two minutes of 5:95 ratio of 5% acetonitrile in water with 0.1 % formic acid and 95% acetonitrile in water with 0.1 % formic acid. This added wash time not only allowed the column to be flushed between samples to keep the column free from analyte build up but this also prevented cross contamination between sample runs as well.

The results obtained in this research show that the method developed was able to detect and quantify gabapentin concentrations in a large range in animal tissue. Given these results, The Ohio State University Neuroscience research group will use the dosage of 200 mg/kg/ day for 8 days giving subcutaneous injections every 2 hours after the last injection of gabapentin method in a future, full scale

gabapentin experiment to characterize any healing properties GBP could have in post spinal cord injury cases and prevent future onset of AD.

Further, more detailed experiments performed by the Neuroscience department will require a more vigorous calibration curve and quantification method. Future work in the analytical development will include a calibration curve for gabapentin created in blank tissue samples rather than just solutions as done in this step of the research. This calibration in tissue will provide a comprehensive picture of any matrix effects that may be present such as if the gabapentin takes on a sodium or potassium ion as opposed to a hydrogen ion when being analyzed. If this occurs, other mass peaks will need to be considered when quantifying the gabapentin in the tissue samples. This calibration in tissue concentrations will also be tested in triplicates across various days/ hours to obtain a more accurate calibration curve to use in a large scale experiment.

At this stage, the research project was to explore dosage and delivery methods as compared to one another, such that this vigorous of a calibration curve was not necessary. Using the calibrations in solution, the quantification method was able to show a seven to ten fold drug presence (on average) in the 200 mg/kg/ day for 8 days at 2 hours after last injection gabapentin as compared to the other three dosage and delivery methods examined. This shows a clear difference in drug presence in the spinal cord given a different dosage and delivery method.

Further work will also include quantifying gabapentin in other tissue samples from the mice. In addition to spinal cord samples, brain, kidney, and spleen samples will be tested for gabapentin concentrations as well. This will give neuroscientists a better look at how the drug is affecting other body parts in the subject's system. Side effects such as nausea, diarrhea, constipation, and flu like symptoms have been reported with the use of gabapentin for seizure control and neuropathy.[32] There have even been reports of encephalopathy associated with gabapentin administration.[33] These side

effects suggest that gabapentin is interacting with other systems outside the spinal cord and brain and it is an important part of the overall picture of how the drug is processed inside the body to know what other systems may be up taking the drug. It is also important to know if harmful concentrations are being achieved in different tissues within the body as well as seeing if interactions with other systems may help or harm the overall healing process.

It will also be during this large scale experiment that neuroscientists will be documenting the progress of the healing process and if gabapentin does in fact help, hinder, or is neutral in the prevention of AD. Knowing the quantity of gabapentin in the possible successful versus unsuccessful cases will help neuroscientists alter and perfect the dosage and delivery recommendations for gabapentin use in preventing future cases of AD in spinal cord injury patients.

References

1. D. Chadwick, Gabapentin. *Lancet* **1994**, 343, 89.

2. M.C. Walker, P.N. Patsalos, Clinical Pharmacokinetics of New Antiepileptic Drug. *Pharm. Ther.* **1995,** 67, 351-384.

3. N.B. Finnerup, H. Gottrup, T.S. Jensen, Anticonvulsants in Central Pain. *Exp. Opin. Pharmacother*. **2002**, 3, 1411-1420.

4. Eroglu, et al. The Gabapentin Receptor α2δ-1 is the Neuronal Thrombospondin Receptor Responsible for Excitatory CNS Synaptogenesis. *Cell* **2009**, 139, 380-392.

5. N.S. Gee, J.P. Brown, V.U. Dissanayake, J. Offord, R. Thurlow, G.N. Woodruff. The Novel Anticonvulsant Drug, Gabapentin (Neurontin), Binds to the Alpha2Delta Subunit of a Calcium Channel. *J Biol Chem*. **1996**,271 (10), 5768-5776.

6. L.C. Weaver, J.C. Fleming, C.J. Mathias, A.V. Krassioukov. Disordered Cardiovascular Control after Spinal Cord Injury. *Handb. Clin. Neurol.* **2012**, 109, 213-233.

7. Krassioukov, V.E. Claydon. The Clinical Problems in Cardiovascular Control following Spinal Cord Injury. *Prog Brain Res.* **2006**, 152, 223-229.

8. E.M. Hagen, Acute complications of spinal cord injuries. *World journal of orthopedics* **2015**, 6 (1), 17-23.

9. A.G. Rabchevsky. Segmental Organization of Spinal Reflexes mediating Autonomic Dysreflexia after Spinal Cord Injury. *Prog Brain Res*. **2006**, 152, 265-274.

10. S. Hou, H. Duale, A.A. Cameron, S.M. Abshire, T.S. Lyttle, A.G. Rabchevsky. Plasticity of Lumbosacral Propriospinal Neurons is Associated with the Development of Autonomic Dysreflexia After Thoracic Spinal Cord Transection. *J Comp Neurol*. **2008**, 509 (4), 382-399.

11. T. Yoshizawa, K. Kadekawa, P. Tyagi, S. Yoshikawa, R. Takahashi, S. Takahashi, N. Yoshimura, Mechanisms inducing autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury. *Spinal Cord* **2015**, 53 (3), 190-194.

12. A.G. Rabchevsky, P.H. Kitzman. Latest Approaches for the Treatment of Spasticity and Autonomic Dysreflexia in Chronic Spinal Cord Injury. *Neurotherapeutics*. **2011**, 8, 274-282.

13. D. Ouellet, H.N. Bockbrader, D.L. Wesche, D.Y. Shapiro, E. Garofalo, Population Pharmacokinetic of Gabapentin in Infants and Children. *Epilepsy Res*. **2001**, 47, 229-258.

14. M.S. Larsen, S. Frolund, M.K. Nohr, C.U. Nielsen, M. Garmer, M. Kreilgaard, R. Holm, In vivo and In vitro Evaluations of Intestinal Gabapentin Absorption: Effect of Dose and Inhibitors on Carrier-Mediated Transport. *Pharmaceutical Research* **2015**, 32 (3), 898-909.

15. Z. Zhu, L. Neirinck, J. Chromatogr, High-Performance Liquid Chromatographic Method for the Determination of Gabapentin in Human Plasma. *Biomed. Appl*. **2004**, 810, 297-303.

16. D.R. Ifa, M. Falci, M.E. Moreas, F.A. Berreza, M.O.Moraes, Gabapentin Quantification in Human Plasma by High-Performance Liquid Chromatography Coupled to Electrospray Tandem Mass Spectrometry. Application to Bioequivalence Study. *J. Mass Soectrom.* **2001**, 36, 188-194.

17. N.V.S. Ramakrishna, K.N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, J. Chidambara, B. Sumatha, D.P. Varma, Rapid Quantification of Gabapentin in Human Plasma by Liquid Chromatography/ Tandem Mass Spectrometry. *J. Pharm. Biomed. Abal*. **2006**, 40, 360-368.

18. C.E. Wolf, J.J. Saady, A. Polkis, Determination of Gabapentin in Serum Using Solid-Phase Ectraction and Gas-Liquid Chromatography. *J. Anal. Toxicol*. **1996**, 20, 498-501.

19. S.Y. Chang, F.Y. Wang, Determination of Gabapentin in Human Plasma by Capillary Electrophoresis with Laser-Induced Fluorescence Detection and Acetonitrile Stacking Technique. J. Chromatogr. *B: Biomed. Appl*. **2004,** 799, 265-270.

20. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.38-40.

21. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.43-45.

22. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.46.

23. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.47.

24. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.52.

25. Agilent 6200 Series TOF LC/MS Techniques. Course #R1874A. Presentation slide 24.Figure.

26. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I. 53.

27. Agilent Time-0f-Flight Mass Spectrometry: Technical Overview. Printed in the USA, October 13, **2011**. 5990-9207EN.

28. Agilent 6200 Series TOF LC/MS Techniques & Operations student Manual. Course #R1874A. Printed in August **2012**. Volume I.

29. C.G. Enke. A predictive model for matrix and analyte effects in electrospray ionization of singly-charged ionic analytes. *Anal Chem* **1997**. 69, 4885–4893.

30. A.P. Bruins, J.D. Henion, T.R. Covey. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. *Anal Chem* **1987**. 59, 2642–2646.

31. N.B. Cech, C.G. Enke. The effect of affinity for charged droplet surfaces on the fraction of analyte charged in the electrospray process. *Anal Chem* **2001**. 73, 4632–4639

32. "Gabapentin." *MedlinePlus Drug Information*. U.S. National Library of Medicine, 15 July **2011**. Web.

33. K. Beauvais, A. Disson-Dautriche, A. Jacquin, C. Antoniol, M.L. Martin, Gabapentin-induced encephalopathy. *Clinical neurophysiology: official journal of the International Federation of Clinical Neurophysiology* **2015**, 126 (4), 845-6.

Appendix

Gabapentin positive ion calculated mass ($C_9H_{17}NO_2$) + H^+

	Mass of atom (amu)	Number of atoms	Sum (amu)
Carbon	12.00000	9	108.00000
Hydrogen	1.00783	17	17.13311
Nitrogen	14.00307	1	14.00307
Oxygen	15.99492	2	31.98984
		Total	171.12602
Plus H	1.00783	1	+1.00783
		Total	172.13385
Minus e-	0.00055	1	-0.00055
		Total	**172.13330**

Gabapentin calculated mass assignment error

$$\frac{(172.1348 - 172.1333) * 1000000}{172.1333} = 8.714177\ ppm\ error$$

Gabapentin standard deviation of the mass measurement

$$s = \frac{10^6}{(2.4 * R * \sqrt{n}\,)}$$

s= standard deviation of the mass measurement
R= resolving power (10,000 for LC/MS TOF)
n = number of ions detected in mass peak

$$0.004883913 = \frac{10^6}{(2.4 * 10000 * \sqrt{72784960.37}\,)}$$

Caffeine positive ion calculated mass ($C_8H_{10}N_4O_2$) + H^+

	Mass of atom (amu)	Number of atoms	Sum (amu)
Carbon	12.00000	8	96.00000
Hydrogen	1.00783	10	10.07830
Nitrogen	14.00307	4	56.01228
Oxygen	15.99492	2	31.98984
		Total	194.08042
Plus H	1.00783	1	+1.00783
		Total	195.08825
Minus e-	0.00055	1	-0.00055
		Total	**195.08770**

Caffeine calculated mass assignment error

$$\frac{(195.0892 - 195.0877) * 1000000}{195.0877} = 7.688850\ ppm\ error$$

Caffeine standard deviation of the mass measurement

$$s = \frac{10^6}{(2.4 * R * \sqrt{n}\,)}$$

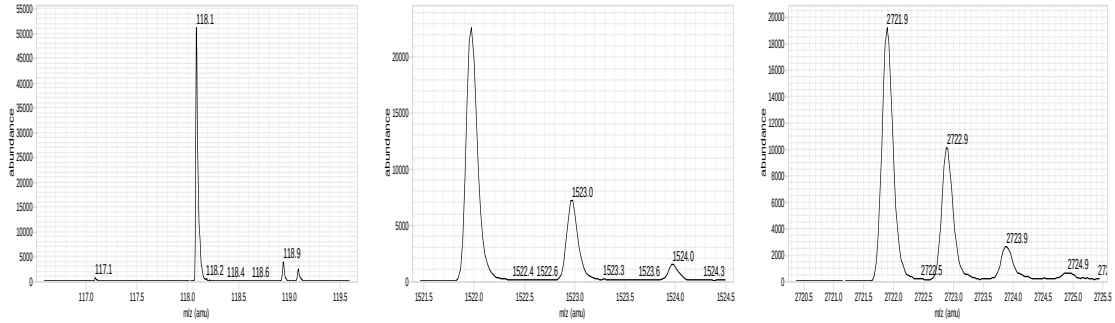
s= standard deviation of the mass measurement
R= resolving power (10,000 for LC/MS TOF)
n = number of ions detected in mass peak

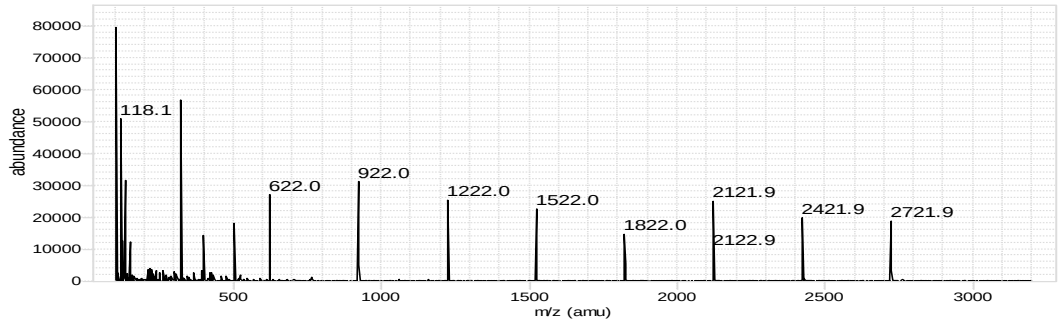
$$0.0093979 = \frac{10^6}{(2.4 * 10000 * \sqrt{19656894.83}\,)}$$

TOF Tune Report

Tune Type:	**TOF Tune**		
MS Model	G6230A	**Tune Date & Time**	**03/18/2015 10:25:12**
Instrument ID		**Instrument State**	Ext Dynamic Range (2 GHz)
Serial#	SG11050201		
Firmware Rev.	4.584		
Data Path	D:\MassHunter\Tune\TOF\Autotune.tun		
Source Type	AJS ESI	***Polarity***	Positive

TOF Results





Calibration Data Table

m/z	Actual	Time	Abund	Res	FWHM	Delta (m/z)	Delta (PPM)
118.086255	118.086255	20.00125	63449	5734	0.0217	0	0
322.048121	322.048136	32.36394	63375	8486	0.0392	0.000015	0.05
622.02896	622.028834	44.57981	35175	10555	0.0603	-0.000126	-0.2
922.009798	922.010136	54.05267	38501	11724	0.0801	0.000338	0.37
1221.990637	1221.990362	62.07291	32216	12245	0.1015	-0.000275	-0.22
1521.971475	1521.971366	69.15545	28368	12740	0.1213	-0.000109	-0.07
1821.952313	1821.952344	75.56806	19285	13100	0.141	0.000031	0.02
2121.933152	2121.933633	81.47114	31039	13194	0.1629	0.000481	0.23
2421.91399	2421.913484	86.96973	24976	13440	0.1823	-0.000506	-0.21
2721.894829	2721.894981	92.13709	24361	13473	0.2043	0.000152	0.06

Setpoints

Parameter	Setting	Actual
Source		
Gas Temp	325	325
Drying Gas	5	5
Nebulizer Pressure	20	20
Capillary	4000	-4000
Sheath Gas Temperature	325	
Sheath Gas Flow	7.5	
Nozzle Voltage	2000	
Optics 1		
Fragmentor	175	
Skimmer	62	
Oct 1 RF Vpp	750	
Oct DC 1	35.8	
Optics 2		
Ion Focus	-160	
Slicer	-9.5	
Horizontal Q	22.8	
Vertical Q	23.6	
Top Slit	9	
Bottom Slit	7.3	

Parameter	Setting
TOF	
Pusher	1250
Puller	-800
Puller Offset	29
Acc Focus	-1880
Front Mirror	-6500
Mid Mirror	-1391.5
Back Mirror	1650
Minimum Mass	100
Maximum Mass	3200
Acquisition Rate	1
Acquisition Time	1000
Length of Transients	199632
#Transients / Spectrum	9894
Low Gain PreAmpOffset	50793
Gain Abund Ratio	12.0
Gain T0 Offset	-0.010
Detector	
MCP	660
PMT	761
PreAmpOffset	39277

Other Actuals:

Parameter	Actual
Rough Vac	1.77
TOF Vac	9.01E-08
Capillary Current	7.009
Chamber Current	3.76
Turbo 1 Speed	100
Turbo 1 Power	175

Calibration Coefficients (in µs)							
a	0.5726004	**a2**	1.068E-06	**c2**	2.7E-09	**e2**	1.357E-15
t0	1.023324	**b2**	-9.41E-08	**d2**	-2.8E-11	**f2**	-4.13E-20
Term Flags:	0x0578	**Trad:**	1	**Poly:**	6		

Calibration Investigation Results